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Early Development of the Japanese Spiny Oyster (*Saccostrea kegaki*): Characterization of Some Genetic Markers

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The phylum Mollusca is one of the major groups of Lophotrochozoa. Although mollusks exhibit great morphological diversity, only a few comparative embryological studies have been performed on this group. In the present study, to begin understanding the molecular development of the diverse morphology among mollusks, we observed early embryogenesis in a bivalve, the Japanese spiny oyster, *Saccostrea kegaki*. Although several studies have begun to reveal the genetic machinery for early development in gastropods, very little molecular information is available on bivalve embryogenesis. Thus, as a step toward identifying tissue-specific gene markers, we sequenced about 100 cDNA clones picked randomly from a gastrula-stage cDNA library. This basic information on bivalve embryology will be useful for further studies on the development and evolution of mollusks.

Key words: *Saccostrea*, embryogenesis, β -tubulin, *tektin*, *vasa*, *frizzled*, *arp2/3*, alkaline phosphatase

INTRODUCTION

Recent molecular phylogenetic studies have classified bilateral triploblastic animals into three groups: lophotrochozoans, ecdysozoans, and deuterostomes (Aguinaldo et al., 1997). Most modern developmental biological studies are devoted to the latter two groups; *Drosophila* and *C. elegans* are representative ecdysozoans, and vertebrates, ascidians, and sea urchins are well-studied model animals among deuterostomes. In contrast, less attention has been paid to lophotrochozoans. It is only in the last decade that some representative species of gastropod mollusks and annelids have been the subjects of molecular developmental studies. However, considering the morphological diversity of lophotrochozoans, there are many interesting animals in this group that have not been examined by modern developmental biology.

Mollusca is a prominent lophotrochozoan phylum whose members exhibit an extensive range of morphological variation. Mollusks are characterized by their calcite skeleton. Shell morphologies show great diversity, which is reflected by classification into seven classes: Aplacophora, Polyplacophora, Monoplacophora, Gastropoda, Bivalvia, Scaphopoda, and Cephalopoda. The phylogenetic relationships among these mollusk classes remain uncertain. Morphological studies have proposed that the aplacophorans are the most primitive group of mollusks, followed by the diver-

gence of Polyplacophora and the conchifera (Salvini-Plawen and Steiner, 1996; Wingstrand, 1985); the latter includes the Monoplacophora, Gastropoda, Cephalopoda, Bivalvia, and Scaphopoda. In the conchifera, phylogenetic affinities between gastropods and cephalopods and between bivalves and scaphopods have been generally accepted by most authors (e.g., Brusca and Brusca, 2003), although Waller (1998) has proposed a phylogenetic affinity among scaphopods, gastropods, and cephalopods. Despite significant efforts, most molecular phylogenetic studies have failed to resolve the phylogenetic relationships among molluscan groups (Passamaneck et al., 2004; Winnepeninckx et al., 1996). The phylogenetic affinity between scaphopods and bivalves (diasome concept) has not been supported by molecular phylogenetic studies (Passamaneck et al., 2004). Therefore, the phylogeny of molluscan classes remains largely unresolved.

Compared with gastropods, for which several genes expressed early in embryogenesis have been identified, bivalves have been little studied ever since Lillie (1895) and Meisenheimer (1901) traced the cell lineages of two bivalve species. These authors described bivalves as having a cell lineage similar to that of gastropods. For example, trochoblast cells are derived from four sets of cells (1q2; q=a, b, c, d), and there are two distinct lineages of mesodermal cells: the anterior mesoderm from 2a (designated as the Y blastomere) and the posterior mesoderm from 4d (designated as the M blastomere). The shell gland is derived from the 2d lineage in both bivalves and gastropods (Collier, 1997); but see also Dictus and Damen (1997). Interestingly, although the cell lineage is comparable, the shell gland cells of bivalves show a unique pattern of cleavage, which is

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reflected in the subsequent shell morphology. After dividing along the anterior-posterior axis four times, the large blastomere of the presumptive shell gland (blastomere X) divides bilaterally, and each daughter cell produces a shell plate bilaterally. Therefore the development of bilaterally separated shell plates, which are characteristic of bivalves, is tightly linked to the cleavage pattern during early embryogenesis. However, the description by Lille (1985) and Meisenheimer (1901) has not been reexamined using modern techniques. To combine information on cell lineages with data obtained by using modern techniques such as gene expression data, we need proper molecular markers to characterize the blastomeres. In this study, we collected some basic information for an analysis of early embryogenesis in bivalves. We observed morphogenesis in Japanese spiny oysters by light microscopy and scanning electron microscopy (SEM). In addition, we sequenced about 100 cDNA clones randomly selected from a gastrula-stage cDNA library (Oda et al., 2002), providing a starting point for identifying tissue-specific gene markers.

MATERIALS AND METHODS

Collection of embryos

Adult oysters (*Saccostrea kegaki*) were collected on the shores around Seto Marine Biological Laboratory, Kyoto University, Wakayama, Japan and around Shimoda Marine Research Center, University of Tsukuba, Shizuoka, Japan. Mature gametes were obtained by dissection and treated with 1 μ M serotonin (serotonin-creatine sulfate complex [Sigma] dissolved in filtered sea water [FSW]) to promote egg maturation. Embryos were fertilized with dissected sperm solution and cultured in filtered seawater at 27°C. The proper density of larvae in sea water is critical for normal development of the swimming gastrula into a D-shaped larva. We usually transferred swimming gastrulae, at about 6 h after fertilization, into fresh FSW at a density less than 100 larvae/ml.

In-situ hybridization

Digoxigenin-labeled RNA probes were synthesized in vitro from cDNA clones by using SP6 RNA polymerase (Invitrogen) and DIG RNA labeling mix (Roche). The embryos were fixed in 4% paraformaldehyde, 0.1 M MOPS (pH 7.5), 2 mM EGTA, and 0.5 M NaCl and stored in 80% ethanol at -20°C. In-situ hybridization was performed following the protocol for ascidian embryos (Yasuo and Satoh, 1994), except that the RNase treatment was omitted during the washing process. In brief, after rehydration, the embryos were treated with 2 μ g/ml proteinase K for 20 min and then post-fixed in 4% paraformaldehyde. After prehybridization, the embryos were hybridized with digoxigenin-labeled probe at 55°C (hybridization buffer: 50% formamide, 6 \times SSC, 5 \times Denhart's solution, 100 μ g/ml yeast RNA, and 0.1% Tween 20). Excess probe was removed by washing the embryos twice in 50% formamide, 4 \times SSC, and 0.1% Tween 20; twice in 50% formamide, 2 \times SSC, and 0.1% Tween 20; and twice in 50% formamide, 1 \times SSC, and 0.1% Tween 20. The embryos were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody, and positive immunoreactions were visualized using NBT/BCIP (Roche). Some of the control sense probes produced a strong non-specific signal at the edge of the shell plate (Fig. 5F). This signal was easily distinguished from a positive signal, because the non-specific signal was observed outside the surface (the RNA probe probably bound to the extracellular matrix secreted from the shell gland), whereas a positive signal was always observed in the cytoplasm.

SEM

Just before fixation, the vitelline membrane was removed from the embryo as follows. The embryos were washed twice in FSW

containing 2 mM EGTA; the vitelline membrane was digested for 10 min with 1% actinase (Kaken-yaku) in FSW containing 2 mM EGTA; and the embryos were washed in FSW.

The embryos without vitelline membrane were fixed in 1% paraformaldehyde and 1% glutaraldehyde in PBS for 3 h at room temperature, or overnight at 4°C. The fixed specimens were washed in PBS, and stored in PBS with 0.1% sodium azide at 4°C. The specimens were dehydrated with a graded ethanol series, immersed in absolute *t*-butanol three times for 20 min each, and then placed in a refrigerator (4°C). The frozen specimens were processed in a Hitachi ES-2030 freeze drier. The dried specimens were mounted on a stub, coated with Pt-Pd, and observed at 10 kV with a Hitachi S-4300 scanning electron microscope.

Histochemistry

The embryos were fixed in 4% paraformaldehyde in PBS for 1 h, washed twice in PBS, and stored in PBS with 0.1% sodium azide at 4°C. Just before staining, the fixed specimens were decalcified in PBS containing 0.05 or 0.1 M EDTA for 30 min and washed three times in PBS.

For alkaline phosphatase staining, the specimens were incubated two times for 10 min each in 0.1 M Tris-HCl, pH 7.5, 100 mM NaCl, and 50 mM MgCl₂, and the reaction was visualized using NBT-BCIP. For phalloidin staining of fibrillar actin, the embryos were placed into rhodamine-conjugated phalloidin (Molecular Probe) diluted 200 fold in PBS, for at least 2 h at room temperature, or overnight at 4°C.

Sequencing of randomly selected cDNA clones

We randomly chose 106 clones from a gastrula-stage cDNA library (Oda et al., 2002) and sequenced the 5' ends of these clones. For some clones, the sequence of the entire insert was obtained.

RESULTS

General description of early development in *Saccostrea kegaki*

The unfertilized eggs released from the gonads are triangular in shape (Fig. 1A) and arrest at the first prophase of meiosis. One end of the egg contains clearer cytoplasm. This region of the oocyte, called the stalk by Pipe (1987), is connected to follicle cells and the ovarian wall during oogenesis. Upon treatment with serotonin, which initiates meiosis, the germinal vesicle soon disappears (Fig. 1B). The eggs become round after their release into sea water (Fig. 1C). The diameter of the fertilized egg is approximately 40 μ m. About 60 min after fertilization, the polar lobe begins to form (Fig. 1D). Within 10 min after the polar lobe appears, the first cleavage begins (Fig. 1E). The polar lobe is incorporated into one of the cells, and the subsequent embryo consists of two cells of very different size (Fig. 1F–H). Polar lobe formation occurs again in the following cell division (Fig. 1I–K), producing a single large blastomere (D blastomere) and three smaller blastomeres of similar size, referred to as the A, B, and C blastomeres (Fig. 1L, Fig. 2A). After the four-cell stage, each blastomere divides asynchronously in a spiral manner (Fig. 2A–C). It is noteworthy that the nomenclature of the blastomeres is not identical between Lillie (1895) and Meisenheimer (1901). Here, we follow the nomenclature of Lillie (1895) because it matches the standard nomenclature for spirally cleaving embryos, established by Wilson (1892) and Conklin (1897).

At 5 h post-fertilization (hpf), ciliary cells are observed in four clusters of four cells each, forming the presumptive pro-

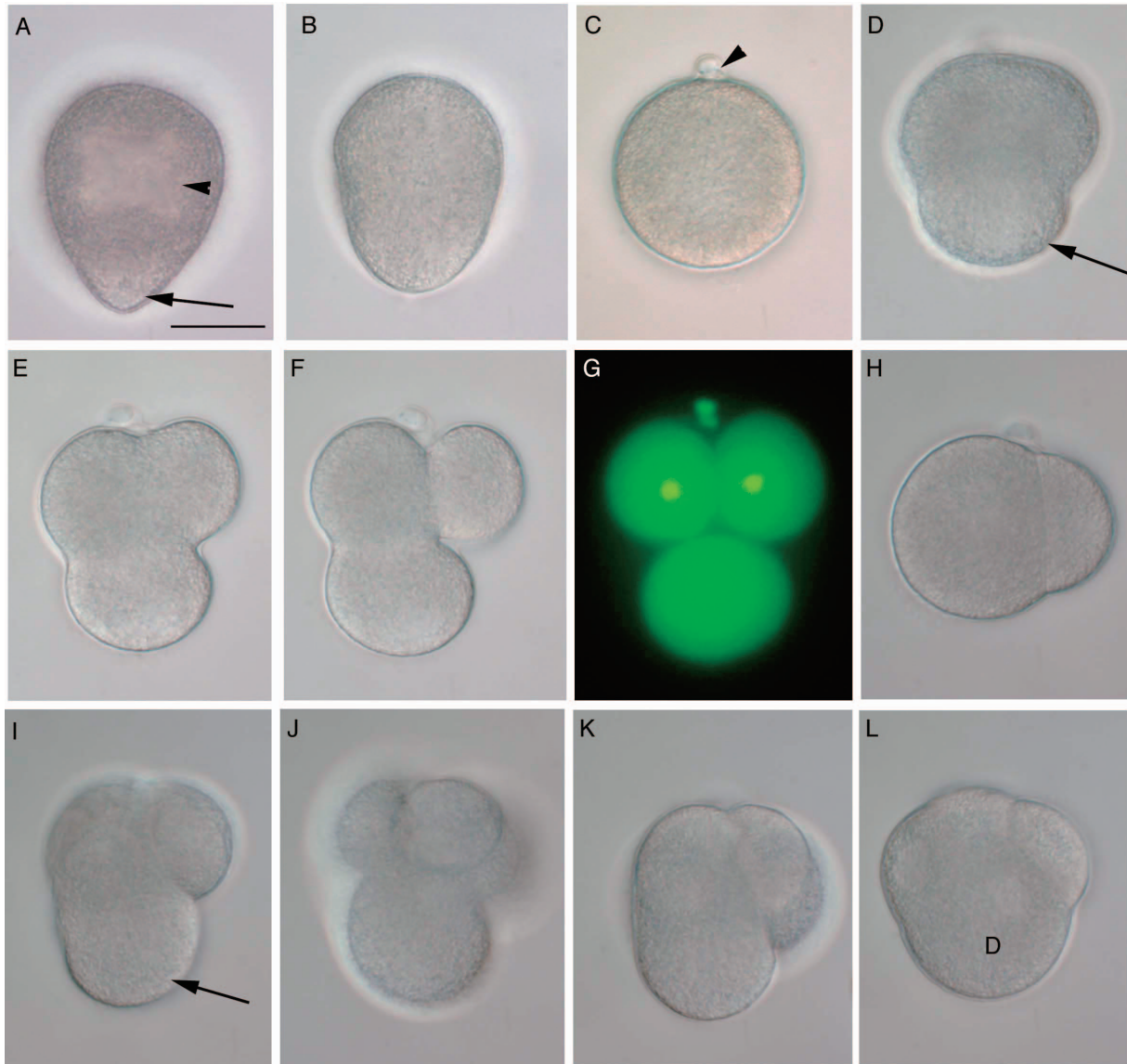


Fig. 1. Early embryogenesis of *S. kegaki* up to the four-cell stage. **(A)** Unfertilized egg. The germinal vesicle is evident (arrowhead). The stalk region of the egg contains clear cytoplasm (arrow). **(B)** After initiation of meiosis by serotonin treatment, the germinal vesicle breaks down. **(C)** Fertilized egg with pole body (arrowhead). **(D)** The polar lobe begins to form from the vegetal region (arrow). **(E)** Soon after polar lobe formation, a cleavage plane begins to form. **(F, G)** After the first cleavage, the egg appears superficially to consist of three cells, although the polar lobe does not contain a nucleus. The nucleus is visualized with Syto-16 in (G). **(H)** The polar lobe is incorporated into one of the blastomeres (CD blastomere). After the polar lobe is incorporated, the embryo consists of two cells of very different size. **(I)** Before the second cleavage, the polar lobe reappears (arrow). **(J)** The second cleavage begins in the animal region. **(K)** After the second cleavage, the polar lobe begins to be incorporated into one of the blastomeres (D blastomere). **(L)** After incorporation of the polar lobe, the embryo has four cells, one of which (D blastomere) is larger than the others. Scale bar=20 μ m.

totroch (Fig. 2D). Shell-gland invagination and gastrulation begin at about 6 hpf. At 8 hpf, the shell-gland invagination and blastopore are clearly observed in the dorsal epidermis and in the vegetal end, respectively (Fig. 2E–G). The shell gland forms as a slit, whereas the blastopore forms as a pit (Fig. 2E, F). At 13 hpf, the shell field begins to grow in a bilateral ribbon shape (Fig. 2H, I). At this stage, a circular ciliary band, the prototroch, is clearly observed (Fig. 2H). In the 16-hpf larva, the shell field has grown to cover the soft body. Anterior and posterior mesodermal cells are clearly visible on the archenteron (Fig. 2J). Telotrochs appear at

the posterior end of the embryo. At this stage, the shell plate divides laterally with the formation of a hinge on the dorsal midline, which characterizes bivalve shells (Fig. 2K). At 18 hpf, the embryo develops into an early D-shaped larva. The D-shaped shell has formed completely (Fig. 2L).

Histochemical staining of the embryo

To characterize tissue differentiation in the D-shaped larva of *S. kegaki*, we examined alkaline phosphatase activity. Alkaline phosphatase was detected in the gut cells of the 24-hpf larva (Fig. 3A). In the 36-hpf larva, an additional

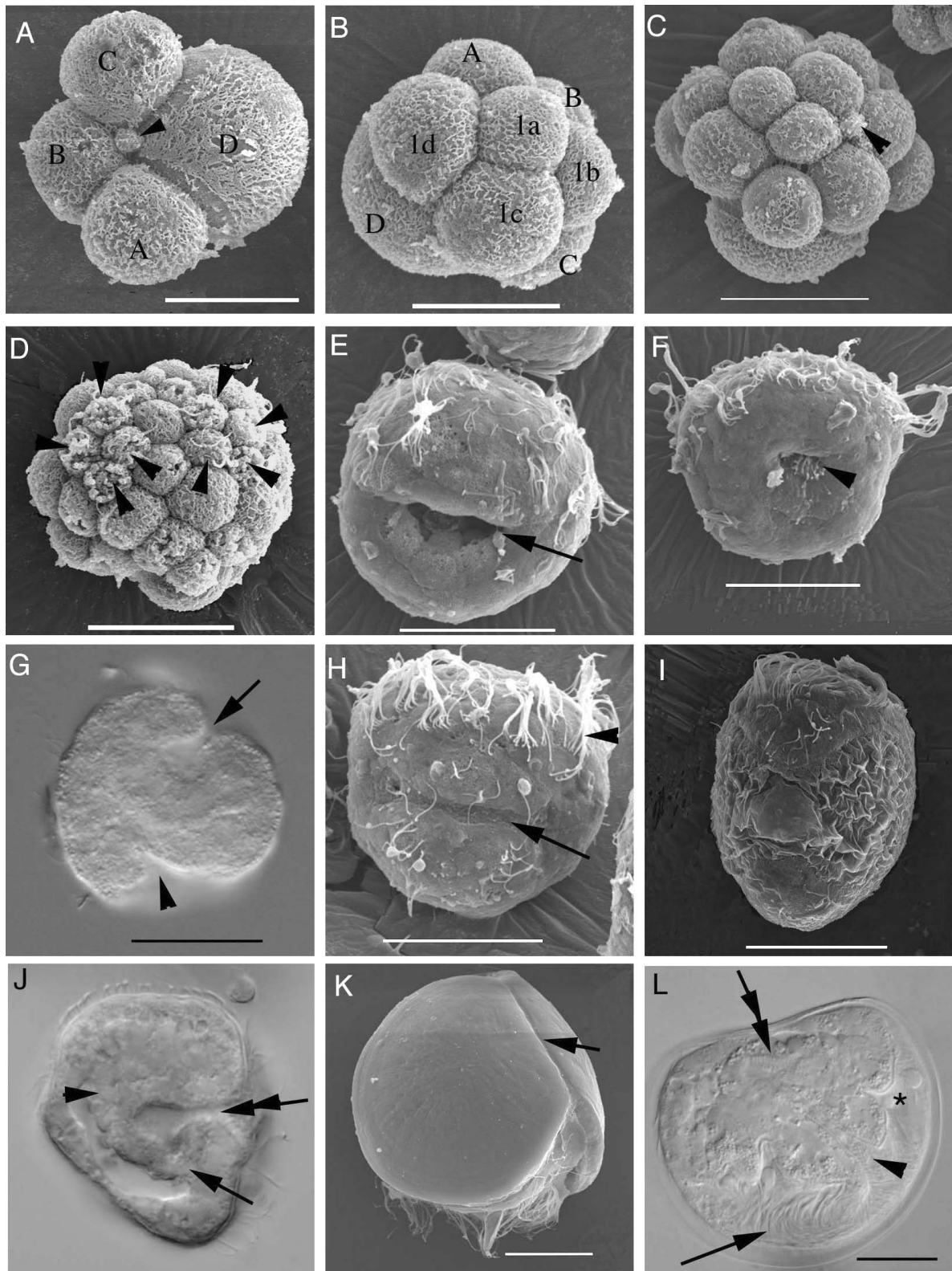


Fig. 2. Embryogenesis of *S. kegaki*. **(A)** Animal view of the four-cell embryo. Arrowhead indicates pole body. **(B)** Animal view of the eight-cell embryo. **(C)** The sixteen-cell embryo. Arrowhead indicates pole body. **(D)** At about the 40-cell stage, the primary trochoblast begins to form ciliary cells; arrowheads indicate pole bodies. **(E–G)** At 8 hpf, the shell gland begins to be seen as a slit (arrow), and the blastopore forms as a pit (arrowhead). **(H)** At 13 hpf, the shell gland begins to evaginate like a ribbon (arrow). An arrowhead indicates the prototroch. **(I)** At 14 hpf, the shell field is visible as a bilateral ribbon. **(J)** At 16 hpf, the anterior (arrowhead) and posterior (arrow) mesoderm cells are visible on the archenteron (double arrow). **(K)** The D-shaped larva with a hinge (arrow) develops at 18 hpf. **(L)** The 24-h larva. The prototroch (arrow), mouth (arrowhead), and anus (asterisk) are visible. Ciliary cells in the stomach are indicated by the double arrow. Scale bar=20 μm.

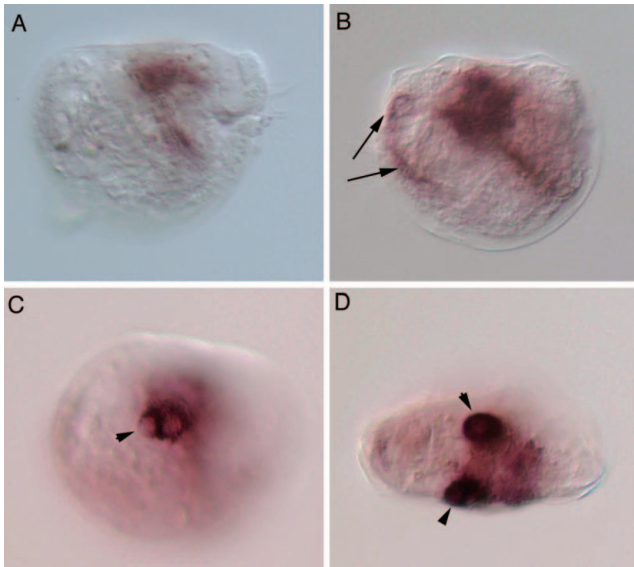


Fig. 3. Alkaline phosphatase activity in larvae. **(A)** In the 24-h larva, alkaline phosphatase activity is detected in endodermal cells (pharynx and stomach). **(B)** In the 36-h larva, an additional signal is observed in the apical region (arrows). **(C, D)** Bilateral clusters of cells are also positive for alkaline phosphatase (arrowheads). Lateral view (C) and dorsal view (D) of the larva; anterior to the left.

signal was observed in the apical region (Fig. 3B). In the 2-day-old larva, two bilateral clusters of cells were also positive for alkaline phosphatase activity (Fig. 3C, D). These cells were just beneath the shell matrix, but we could not identify them.

To examine muscle cell differentiation, phalloidin was used to visualize actin fibers. Adductor muscle cells were observed in the 15-hpf larva (data not shown). In the 24-hpf larva, four pairs of retractor muscle bands appeared (Fig. 4): two pairs were attached to the pretrochal region, and the posterior two pairs were attached to the posttrochal region.

Sequencing of randomly selected cDNA clones

To look for tissue- or cell type-specific markers in oyster embryogenesis, we sequenced the 5' ends of 106 clones selected randomly from a gastrula-stage cDNA library (Oda et al., 2002). Among the 106 clones, 46 independent transcripts (from 49 clones read) showed significant similarity to known sequences in the Uniprot database (the cut-off value was less than $1e^{-5}$; Table 1). We chose five of these genes for expression analysis by in-situ hybridization.

Expression of *Sk- β -tubulin*

No maternal expression was detected for *Sk- β -tubulin*. The first indication of gene expression was detected at about the 24-cell stage, in the primary trochoblasts (Fig. 5A). At about the 40-cell stage, expression was maintained in the primary trochoblasts (Fig. 5B). In the late gastrula (12 hpf), all of the ciliated cells of the circular ciliary band were positive for *Sk- β -tubulin* expression (Fig. 5C, D). In the early D-shaped larva (20 hpf), expression was detected in the ciliary band and the telotrochs (Fig. 5E). Some cells in the gut were also positive for *Sk- β -tubulin* expression at this stage (Fig.

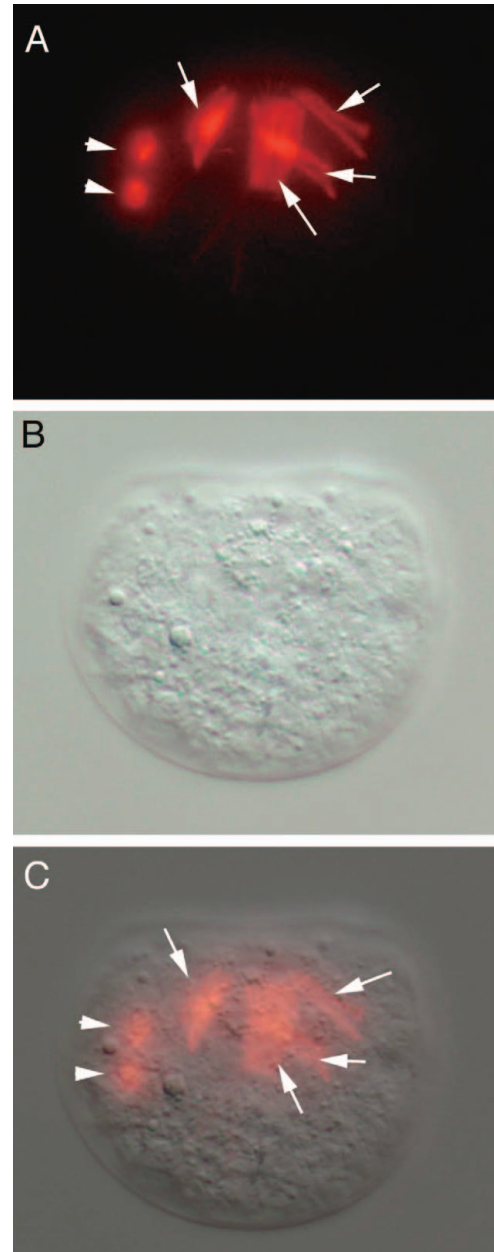


Fig. 4. Fibrillar actin in the D-shaped larva visualized by phalloidin. Four pairs of retractor muscle bands are visible (arrows), each of which possesses two separated fibers in the 24-h D-shaped larva. Adductor muscle (arrowheads) is also visible in the anterior part of the larva. **(A)** Actin fibers visualized by rhodamine-phalloidin fluorescence. **(B)** Light image of the larva. **(C)** Phalloidin signal overlaid on the light image (dorsal to the top, anterior to the left).

5E), which was consistent with the presence of cilia in the stomach (Fig. 1L).

Expression of *Sk-tektin*

The expression of *Sk-tektin* resembled that of *Sk- β -tubulin*, although the earliest expression was detected slightly later than that of *Sk- β -tubulin*. The earliest expression of *Sk-tektin* was detected at about the 40-cell stage, whereas that of *Sk- β -tubulin* was at the 24-cell stage.

Table 1. Characterization of sequenced clones from a gastrula cDNA library.

Best hit genes	best hit genes from	best hit gene Acc. No.	Saccostrea gene Acc. No.
Transcription factors/nuclear proteins			
ICBP90 protein	Homo sapiens	AF129507	AB375019
HMG protein Tcf/Lef protein	Strongylocentrotus purpuratus	AF161594	AB375020
High mobility group protein 1 (HMG-1)	Gallus gallus	P36194	AB374934**
histone H2 A.F/Z protein	Strongylocentrotus purpuratus	X05547	AB375021
ring finger protein 121 protein	Mus musculus	BC022686	AB375022
MOP-3 protein	Homo sapiens	AB014772	AB375023
TIP120 protein	Rattus norvegicus	D87671	AB375024
nucleolar protein ANKT protein	Mus musculus	AF305710	AB375025
histone 1 protein	Mytilus edulis	AJ224077	AB375026
NUCLEAR PROTEIN HCC-1 homolog	Mus musculus	AK018773	AB375027
RNA binding protein			
VASA protein	Oryzias latipes	AB063484	AB374933**
Cytoskeleton			
tektin C1 protein	Strongylocentrotus purpuratus	U38523	AB374930*
Tara protein	Homo sapiens	AF281030	AB375028
actin related protein 2/3 complex, subunit 1A protein	Mus musculus	BC001988	AB374931*
alpha-1 tubulin protein	Hirudo medicinalis	U67677	AB375030
beta-tubulin	Monosiga brevicollis	AY026071	AB374929*
Cellular metabolism			
cyclin A	Dreissena polymorpha	AF086635	AB375031
cyclin A	Patella vulgata	X58357	AB375032
Elongation of very long chain fatty acids protein 4	Macaca fascicularis	Q95K73	AB375033
carbonic anhydrase 2 protein	Tribolodon hakonensis	AB055617	AB375034
farnesyl diphosphate synthase protein	Dictyostelium discoideum	AF234168	AB375035
Tat-binding protein-1 protein	Drosophila melanogaster	AF134402	AB375036
RE08109p protein(peptidyl-prolyl cis-trans isomerase)	Drosophila melanogaster	AY070988	AB375037
carnitine palmitoyltransferase I protein	Sus scrofa	AF288789	AB375038
cytochrome oxidase subunit I protein	Saccostrea cucullata	AY038076	AB375039
growth arrest specific 11 protein	Homo sapiens	AF050079	AB375040
cyclin B3 protein	Xenopus laevis	AJ304990	AB375041
thioredoxin-like protein DPY-11 protein	Caenorhabditis elegans	AF250045	AB375042
checkpoint suppressor 1 protein	Mus musculus	AK17346	AB375043
ribosomal protein S4 protein	Argopecten irradians	AF526210	AB375044
Receptor/cell adhesion protein			
neuromedin U receptor type 2 protein	Mus musculus	AY057384	AB375045
Frizzled-1 precursor	Xenopus laevis	Q9I9M5	AB374932**
Ubiquitin pathway			
polyubiquitin protein	Homo sapiens	D63791	AB375046
polyubiquitin protein	Homo sapiens	D63791	AB375047
ubiquitin-conjugating enzyme protein	Drosophila melanogaster	X62575	AB375048
Other functions/Unkown function			
uteroferrin protein	Sus scrofa	M98553	AB375049
CG5325-PA, isoform A protein	Drosophila melanogaster	AE003635	AB375050
FYVE and coiled-coil domain containing 1 protein	Homo sapiens	AJ292348	AB375051
RIKEN cDNA 2700078E11 gene protein	Mus musculus	BC026363	AB375052
CG2616-PA protein	Drosophila melanogaster	AE003676	AB375053
neuronal leucine-rich repeat protein protein	Xenopus laevis	AB014462	AB375054
Hypothetical protein F55F3.1 protein	Caenorhabditis elegans	Z81550	AB375055
hypothetical protein FLJ22167 protein	Homo sapiens	BC010609	AB375056
NCLN protein	Homo sapiens	BC025926	AB375057
COP9 signalosome subunit 8 CSN8 protein	Homo sapiens	U51205	AB375058
RIKEN full-length enriched library, clone:2310005N01	Mus musculus	AK009177	AB375059
Sequence 75 from Patent WO0218424	Homo sapiens	AX399904	AB375060

*Clones used for in-situ hybridization. Full insert sequences were determined for these clones.

**Clones used for in-situ hybridization. Full coding regions were not included in the clones, although full insert sequences were determined.

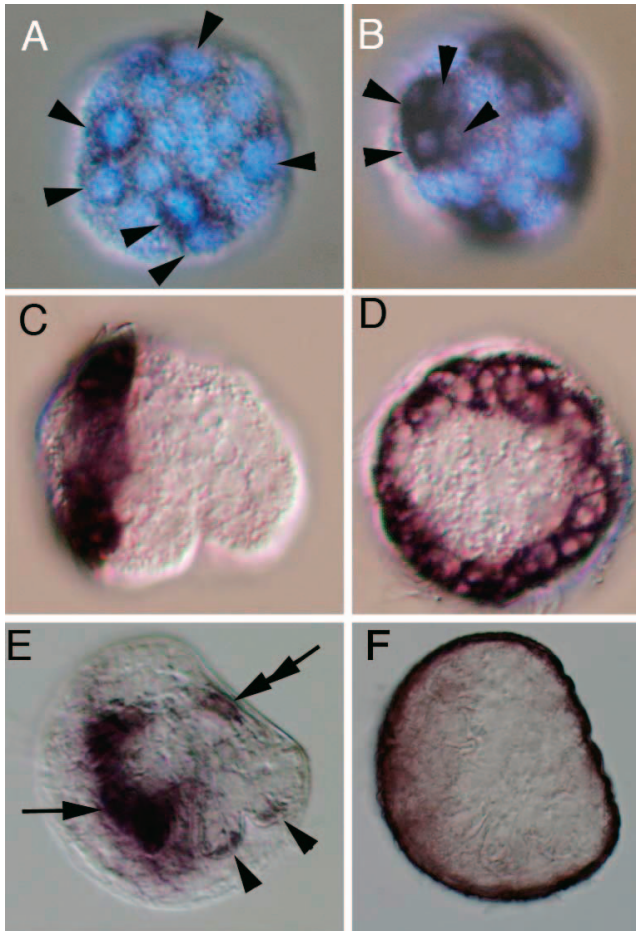


Fig. 5. Expression of *Sk-β-tubulin*. **(A)** In the 24-cell stage, expression is detected in the primary trochoblasts (arrowheads). The nucleus is visualized by DAPI staining. At this stage, the trochoblast consists of four clusters of two cells each. **(B)** At about the 40-cell stage, expression is maintained at the primary trochoblasts, with four cells in each cluster (arrowheads). **(C, D)** In the late gastrula (12 hpf), *Sk-β-tubulin* is detected in the ciliated cells of the circular ciliary band. Lateral view **(C)** and anterior view **(D)** of the embryo. **(E)** In the early D-shaped larva (20 hpf), expression is detected at the ciliary band (arrow) and the telotrochs (arrowheads). Expression is also detected in the stomach (double arrow). **(F)** Non-specific staining at the edge of the shell obtained by a sense-strand probe. Anterior views **(A, B, D)** and lateral views **(C, E, F)**; dorsal to the top, anterior to the left.

Expression was detected in the primary trochoblasts (Fig. 6A). In the late gastrula (12 hpf), expression was detected in the circular ciliary band (Fig. 6B, C). In the D-shaped larva (24 hpf), expression was detected in the ciliary band. *Sk-tektin* expression was also observed in the ciliary cells of the stomach (Fig. 6D).

Expression of *Sk-vasa*

Maternal expression of *Sk-vasa* was observed throughout the embryo until the eight-cell stage (data not shown). At about the 50-cell stage, expression was detected in a pair of 2d descendant cells and a pair of cells located posteriorly (Fig. 7A), which were probably 4d lineage cells. In the gastrula (8 hpf), expression was detected in a pair of cells internalized just posterior to the blastopore; posterior mesodermal cells are descendants of 4d (somatoblast, M blastomere; Fig. 7B, C). In the late gastrula (12 hpf), strong expression was maintained in the posterior mesoderm, and weak expression was detected in the endoderm cells (Fig. 7D).

Expression of *Sk-frizzled*

Maternal expression of *Sk-frizzled* was observed throughout the embryo until the four-cell stage (Fig. 8A). At about the 60-cell stage, expression was detected only in the 2d descendant cells (Fig. 8B). This expression disappeared before the gastrula stage.

Expression of *Sk-Arp2/3*

The earliest expression of *Sk-Arp2/3* was detected at the late gastrula stage (12 hpf), in a pair of anterior internalized cells (larval mesoblast, Y blastomere; Fig. 9A, B). In the 16-hpf larva, expression was detected in the dorsal mesenchyme cells (Fig. 9C). Expression was maintained until the 24-hpf D-shaped larva developed (Fig. 9D).

DISCUSSION

Lillie (1895) and Meisenheimer (1901) described the early development of the bivalves *Unio* and *Dreissensia*, respectively. We found that the early embryogenesis of the Japanese spiny oyster, *S. kegaki*, is quite similar to the early development of these two species. All of the species show unequal cleavages and fix the dorsoventral axis before the four-cell stage, although the polar lobe was not observed in

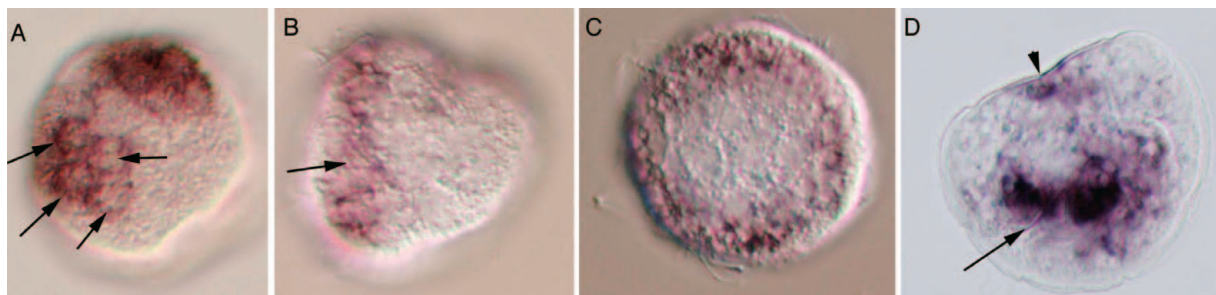


Fig. 6. Expression of *Sk-tektin*. **(A)** At the 40-cell stage, expression is detected at the primary trochoblasts (arrows). **(B, C)** At the late gastrula stage (12 hpf), expression is detected in the circular ciliary band (arrow). Lateral view **(B)** and anterior view **(C)** of the embryo. **(D)** In the D-shaped larva (24 hpf), expression is detected in the ciliary band (arrow) and stomach (arrowhead) (anterior to the left, dorsal to the top).

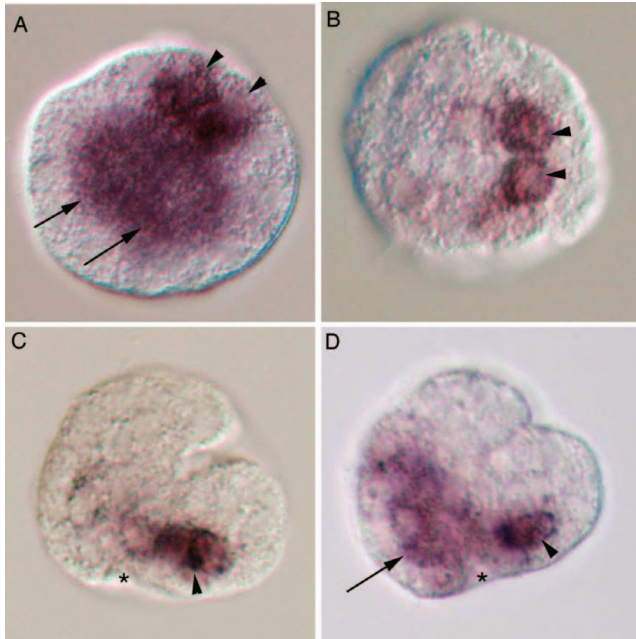


Fig. 7. Expression of *Sk-vasa*. **(A)** At about the 50-cell stage, expression is detected in a pair of 2d descendant cells (arrows) and a pair of 4d lineage cells located posteriorly (arrowheads). **(B, C)** At the gastrula stage (8 hpf), expression is detected in a pair of cells internalized just posterior to the blastopore (asterisk), representing posterior mesodermal cells descendent of 4d (somatoblast, M blastomere; arrowhead). **(D)** In late gastrula stage (12 hpf), weak expression is also observed in the endoderm cells (arrow) together with the expression in somatoblasts (arrowhead). Lateral views (C, D); anterior to the left, dorsal to the top.

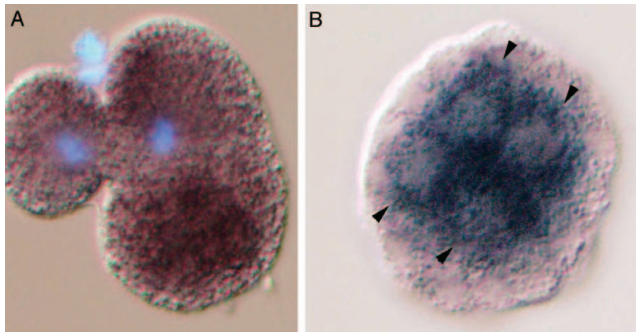


Fig. 8. Expression of *Sk-frizzled*. **(A)** Maternal expression of *Sk-frizzled* is observed throughout the two-cell embryo. The nucleus is visualized by DAPI staining. **(B)** At about the 60-cell stage, expression is detected only in four 2d descendant cells (arrowheads).

Unio or *Dreissensia*. The 2d micromere is the largest cell (Lillie, 1895; Meisenheimer, 1901). As was observed in *Unio* and *Dreissensia*, the shell gland invaginates from the dorsal part of the embryo at almost the same time gastrulation begins (Lillie, 1895).

Markers for ciliary cells

Sk-β-tubulin expression appeared at the 24-cell stage in oyster embryos (Fig. 5A). This is slightly earlier than in

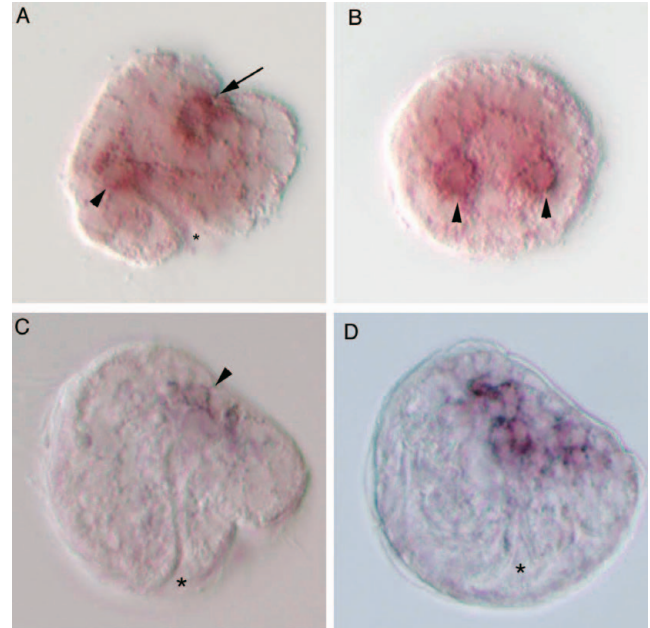


Fig. 9. Expression of *Sk-Arp2/3*. **(A, B)** In the gastrula (8 hpf), expression is detected in a pair of anterior internalized cells (larval mesoblast, Y blastomere; arrowheads). Lateral view (A: anterior to the left) and ventral view (B: anterior to the top) of the embryo. Non-specific staining is observed in the matrix of the shell gland (arrow). The asterisk indicates the blastopore. **(C)** At 16 hpf, expression is detected at the dorsal mesenchyme cells (arrowhead). **(D)** In the 24-h larva, expression is maintained in mesenchymal cells. (C, D) An asterisk indicates the mouth. Lateral views; anterior to the left, dorsal to the top.

Gastropoda, where *β-tubulin* expression first appears at the 32-cell stage of primary trochoblasts (Damen et al., 1994). Primary trochoblast cells form a ciliary band or prototroch. *Sk-tektin* was also expressed in these trochoblast cells, although slightly later, from about the 40-cell stage. Tektin is a filament-forming protein associated with ciliary and flagellar microtubules, and sea urchin *tektin* is expressed in the ciliary band (Norlander et al., 1995). Thus, it is reasonable for it to be expressed in the molluscan ciliary cells. *β-tubulin* and *tektin* are good markers of ciliary cells in bivalves.

Early blastomeres of oyster embryos are difficult to identify simply by observation with light microscopy, because the cleavage pattern is not bilaterally symmetrical and cleavage occurs asynchronously in each blastomere. To identify cells after the 24-cell stage, 2d-derived cells (the largest blastomere) can be used as a landmark. As the other blastomeres are almost the same size, additional landmarks are needed to identify them. *β-tubulin*, which showed strong expression in the anterior part of the embryo, is a good landmark.

Markers for the shell field

Several genes, including *Hox1*, *Hox4*, *engrailed*, and *BMP2/4*, have been characterized in shell formation in gastropods and scaphopods (Jacobs et al., 2000; Moshel et al., 1998; Wanninger and Haszprunar, 2001; Nederbraght et al.,

2002b; Hinman et al., 2002). The expression of *Sk-frizzled* was detected in presumptive shell gland-forming cells (2d). Frizzled is known as a receptor of Wnt proteins (Logan and Nusse, 2004). The 2d-specific expression of *Sk-frizzled* suggests that Wnt signaling plays a role in the early development of molluscan shell fields.

Endoderm markers

Alkaline phosphatase activity is detected in the gut cells of swimming larva and can serve as an endoderm marker as in other invertebrates, including ascidians and sea urchins (Amemiya, 1996; Nishida, 1992). However, it should be noted that an additional signal is observed in the apical region. The other alkaline phosphatase-positive cells, which are located bilaterally beneath the shell plate, remain to be characterized.

Mesoderm markers

Two populations of mesoderm cells have been described in bivalves and gastropods. In gastropods, anterior mesoderm is marked by *twist* and *fork head* (Nederbraght et al., 2002a; Lartillot et al., 2002), and posterior mesoderm is marked by *cdx* (Gounar et al., 2003). In bivalves, anterior mesoderm is derived from 2a (Y blastomere, larval mesoblast), and posterior mesoderm is derived from 4d (M blastomere, somatoblast). We found that in oysters, anterior mesoderm is positive for *Sk-arp2/3*, whereas posterior mesoderm is positive for *Sk-vasa*. Arp2/3 is involved in polymerization and organization of actin filaments (Goley and Welch, 2006). Lillie, (1895) noted that larval muscle cells differentiate from anterior mesoderm cells (Y blastomeres), which is consistent with the expression of *Sk-arp2/3* in anterior mesoderm. Bivalves acquired a novel morphology consisting of separated shell plates. For a separated shell plate to be adaptive, evolution of the adductor muscle may have been essential to close the shell plates. We observed both adductor and retractor muscle bands by visualizing actin microfilaments. The differentiation of these types of muscle from anterior mesoderm precursors (visualized by *Sk-arp2/3*) is an interesting subject that we are currently investigating.

Expression of *Oyvlg*, a *vasa* homolog of another species of oyster, *Crassostrea gigas*, has already been described in the germ cells (Fabioux et al., 2004b). *Oyvlg* mRNAs show localized distribution and are segregated into a specific blastomere (Fabioux et al., 2004a). At the gastrula stage, *Oyvlg* expression is detected in posterior mesodermal cells (4d), from which primordial germ cells have been suggested to originate. We could not detect localized mRNA of *Sk-vasa* in early embryogenesis, which might be because we used a different species, or because our procedure of in-situ hybridization was slightly different. Despite this difference, the expression in the 4d lineage was observed for *Sk-vasa* as well. This expression is consistent with the idea that primordial germ cells originate from 4d (M blastomere) cells (Fabioux et al., 2004a).

Molluscan evolution and bivalve embryology

In this study, we described embryogenesis in a bivalve, approaching molluscan body-plan diversification from the aspect of molecular developmental biology. Recently,

embryogenesis has been described for representatives of Aplousophora, Scaphopoda, and Cephalopoda, and molecular tools have been applied to Scaphopoda and Cephalopoda (Okusu, 2002; Wanninger and Haszprunar, 2001; Lee et al., 2003). On the other hand, studies on bivalve species have been scarce, even though Bivalvia is one of the most species-rich classes of Mollusca. Among the most notable novelties of Bivalvia are the two separate shell plates. The development of separated shell plates is closely linked to the early cleavage pattern of the presumptive shell gland cells. Lillie (1895) and Meisenheimer (1901) described the unique cleavage pattern of the presumptive shell gland cells, which are the earliest cells to show bilateral cell division. This bilateral cell division is closely linked to the morphology of the shell plates, because each daughter cell of the bilateral cleavage develops into cells underlying a single shell plate. Therefore, bivalves acquired the novel shell plate morphology by modifying the early cleavage pattern. However, compared to gastropods, very little information is available on the early embryogenesis of bivalves, especially from the field of molecular biology. We still depend on the descriptions of Lillie (1895) and Meisenheimer (1901), which were written more than 100 years ago. By providing information on molecular markers in the early embryogenesis of bivalves, our study serves as a platform for future studies on the diversification of molluscan body plans.

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